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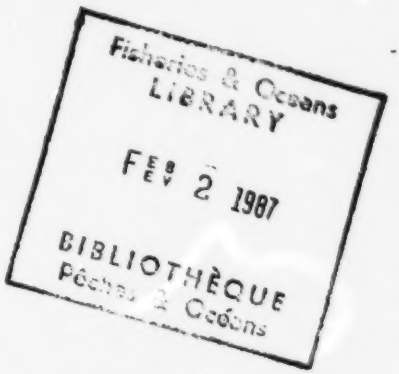

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Characterization of estradiol-specific receptors; induction of the synthesis of vitellogenin and its mRNA in the liver of rainbow trout (Salmo gairdneri)

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Résumé — *Chez toutes les espèces ovipares étudiées à ce jour, la synthèse et l'utilisation de la vitellogénine (Vg) est un phénomène intimement lié à la reproduction. La synthèse de la Vg dans le foie de truite arc-en-ciel (Salmo gairdnerii) est sous la dépendance de l'œstradiol (E_2) qui agit vraisemblablement selon le schéma classique du mécanisme d'action des hormones stéroïdes. Après liaison de l'hormone à un récepteur spécifique, le complexe œstradiol-récepteur peut interagir avec la chromatine et moduler l'expression du gène de la Vg, ce qui se traduit par une augmentation de la synthèse de RNAm Vg et de Vg. Nous montrons ici : (1) la présence de récepteurs spécifiques des œstrogènes (constante de dissociation $K_D \simeq 1{,}5 \times 10^{-9}$ M pour E_2) dans le cytosol du foie de truite mâle; (2) que le foie du male constitue un contrôle expérimental idéal au niveau « zéro », permettant de suivre, après stimulation par E_2, la cinétique d'induction du mRNA Vg par hybridation avec le cDNA Vg; et (3) l'apparition de Vg dans le sérum en utilisant une technique de dosage original (rocket immuno-électrophorèse). Le modèle vitellogénine du foie de truite mâle et les techniques originales mises au point seront très précieuses pour étudier l'influence des facteurs endogènes et exogènes sur les différentes étapes (récepteurs, transcription, traduction) de la régulation de la synthèse de Vg.*

Mots-clés : vitellogénine / œstradiol / récepteurs / RNAm vitellogénine / truite.

Summary — *In all egg laying vertebrates, synthesis and use of vitellogenin (Vg) are intimately bound to the active phase of reproduction. In the liver of the rainbow trout (Salmo gairdnerii), Vg synthesis is influenced by estradiol (E_2) which, we believe, acts through the classical mechanism of steroid hormone action. After binding of the hormone to a soluble specific receptor protein, the estradiol-receptor complex can interact with chromatin and modulate the expression of Vg genes, leading to increased synthesis of specific mRNA and Vg. We show here : (i) the presence of specific œstrogen receptors (dissociation constant $K_D \simeq 1.5 \times 10^{-9}$ M for E_2) in the cytosol of the male trout liver. (ii) The male liver, offering, an ideal experimental control of "zero" background, we followed — in the liver of male trouts — the kinetics of induction of Vg mRNA by hybridization with Vg cDNA, after E_2 stimulation, and (iii) the apparition of Vg in the serum by using an original rocket immuno-electrophoretic technique. The male trouts liver vitellogenin model and the original techniques we developed will be very useful to study the influence of endogenous and exogenous factors on the different steps (receptors, transcription, translation) of vitellogenesis regulation.*

Key-words : vitellogenin / estradiol / receptors / mRNA vitellogenin / trout.

Translator's Note: The above was photocopied from the original French.

INTRODUCTION

In oviparous vertebrates, the vitellus proteins are synthesized in the liver as a large precursor molecule, vitellogenin, which breaks down into phosvitin and lipovitelline after its incorporation into the oocytes [1-4].

The synthesis and utilization of vitellogenin are phenomena that are closely linked to reproduction. As in the other vertebrates, vitellogenin synthesis in fish is controlled directly by estrogens: estradiol [5-10] and estrone [11, 12]. However, aside from work by Roach and Davis [13] on the catfish and more recently by Chen [14] on the rainbow trout, there are hardly any data on the molecular mechanisms for the regulation of the synthesis of this protein in fish.

The objective of this study, using techniques that we have developed or adapted (dosage of vitellogenin and its messenger RNA), was to demonstrate in rainbow trout the presence of the different stages of the molecular mechanism for activating the vitellogenin genes, i.e., binding of estradiol to a specific receptor and induction or modification of the expression of the genes involved in the synthesis of mRNA and vitellogenin.

MATERIALS AND METHODS

Animals and reagents

The rainbow trout (Salmo gairdnerii) were obtained from the fish farm at Gournay/Aronde (Oise), and were kept in the tanks of the Laboratoire de Physiologie des Poissons [fish physiology laboratory] (INRA Rennes) in a recycled water system [15] until the utilization of the trout in the experiment.

The following reagents were obtained from the indicated suppliers: 17β-estradiol (E_2), estrone (E_1), estriol (E_3), diethylstilbestrol (DES), 5α-dihydrotestosterone (5α-DHT), 5α-androstane-3β, 17β-diol (3β-diol), Coomassie blue, ethidium bromide, ficoll, polyvinylpyrrolidone, bovine serum albumin (BSA), salmon testicular DNA (Sigma Chemical Company, Saint Louis, USA). Deoxyribonuclease I, DNA polymerase I, the 4 deoxyribonucleotides dATP, dCTP, dGTP and dTTP (Boehringer Mannheim, West Germany). Deoxy (1',2', 5'-^{3}H) cytidine 5' triphosphate 50 Ci/mmole, methyl (1',2',5-^{3}H) thymidine 5' triphosphate 109 Ci/mmole, (6,7-^{3}H) 17β-estradiol 55 Ci/mmole (Amersham France SA).

Preparation of the cytosol

The cytosol was prepared by homogenizing the liver in 4 mL of buffer I (20 mM Tris, 1.5 mM EDTA, 1 mM DTT, 10 mM

Na_2MoO_4) pH 7.4. The homogenate was centrifuged at 35,000 × g at 0°C for 20 minutes and again at 200,000 × g at 0°C for 90 minutes.

Partial purification of receptors

The fractionated precipitation of the soluble proteins was carried out on a cytosol fraction in $(NH_4)_2SO_4$ (AS) at 30% and 50% saturation. A saturated solution of $(NH_4)_2SO_4$ in buffer I was added one drop at a time, with stirring, to cytosol fractions. After 30 minutes, the solution was centrifuged at 3,600 × g for 10 minutes. The 30% AS and 50% AS pellets were then dissolved in buffer I, in a volume defined by the number of assays to perform.

Measurement of ^3H-E_2 binding

The dissociation constants were determined by adding increasing concentrations of (^3H)-E_2 (from 7.5×10^{-10} M to 5×10^{-8} M) ± a 100-fold excess of (^1H)-E_2 to 75 µL of crude cytosol or of a preparation of partially purified receptors. The final volume of each assay was made up to 250 µL.

The specificity of the binding was determined by adding 75 µL of crude cytosol to 175 µL fractions of (^3H)-E_2 (2.88×10^{-9} M) containing increasing concentrations of steroid competitors in buffer I.

After 3 hours of incubation at 0°C, the amount of radioactive hormone bound to the receptors was measured by adding

25 µL of dextran-charcoal at 10% (DCC) to buffer I at 0°C, centrifuging at 3,600 × g for 10 minutes at 0°C and determining the counts for 150 µL of supernatent in 10 mL of ACS (Amersham). Nonspecific binding was measured by the quantity of bound (^3H)-E_2 in the presence of an excess of (^1H)-E_2. This quantity was subtracted from the other values to obtain the quantity of specifically bound (^3H)-E_2.

Extraction of steroids and thin layer chromatography

After being incubated for 3 hours at 0°C in the presence of (^3H)-E_2 (5×10^{-9} M), the cytosol was extracted by 2 × 5 volumes of a 1:1 cyclohexane-ethyl acetate mixture. After centrifugation for 10 minutes at 3,600 × g, the organic phase was evaporated until dry. The dry extract was redissolved in methanol containing the control steroids E_1, E_2 and E_3 (1 mg/mL) and applied to a 20 × 20 cm silica gel plate. The steroids were then separated at room temperature in a 80:20 chloroform-ethyl acetate solvent system. After noting the positions of the control steroids at 254 and 366 nm, the silica gel was scraped off as fractions measuring 0.5 × 1 cm. The steroids were then extracted from each fraction using 2 × 2 mL of ethyl acetate, evaporated until dry, redissolved in 200 µL of methanol, and the counts determined in 2 mL of ACS.

Extraction of RNAs

The livers are rapidly removed from the trout, frozen in liquid nitrogen and stored in a freezer at -80°C until their use. The total RNA is extracted using the technique of Auffray and Rougeon [16] as modified by Tenniswood and Simpson [17]. The poly $(A)^+$ RNA was prepared by chromatography on oligo-dT cellulose as described by Aviv and Leder [18], with a recovery ratio of 10 to 30 µg of poly $(A)^+$ RNA per mg of total RNA [19]. The RNA preparations were analyzed by electrophoresis on agarose slab gels (1.2%) under denaturing conditions [20] to ensure the quality of the preparations. The RNA concentration was determined by measuring the absorption at 260 nm of an aliquot.

Hybridization of vitellogenin messenger RNA

The RNAs are covalently bound to paper filters treated with aminothiophenol and diazotized as described by Seed [21]. Aliquots (10 µL) containing 10 µg of total RNA or 5 to 10 ng of poly $(A)^+$ RNA are applied to the filters and incubated for 12 hours between 2 sealed sheets of parafilm. Next, the hybridizations are carried out following the conditions described by Wahl *et al.* [22]; the filters are then burned in a Packard Tricarb (model 306) "auto-oxidizer", and the radioactivity is determined by liquid scintillation using an Isocap 300 counter (Nuclear Chicago).

The probe was prepared in collaboration with M Tenniswood, D Bailey and J R Tata (National Institute for Medical Research, Mill Hill, London), and consisted of a pAT 153 plasmid into which was inserted a 0.6 Kb fragment corresponding to the messenger RNA sequence for vitellogenin [23]. This probe was characterized by the Northern blot method (Fig. 1) and was labelled by nick translation using tritiated deoxynucleotide triphosphates. The specific activity ranged from 7 to 12 μCi/μg of DNA.

Dosage of the vitellogenin

Blood samples from the fish in the experimental group were collected by venipuncture of the caudal vein using a heparinated syringe. After being decanted for 24 hours, the serum was obtained by centrifugation at 2,000 × g. The samples were then stored in a freezer at -20°C. The vitellogenin level was then determined using an immunoelectrophoresis technique that we developed after a specific antibody was prepared. Plastic films (FMC Gelbond films) were cut into 5 × 5 cm squares. The antivitellogenin antibody was diluted to 1/400 in a 1% agarose solution. After the agarose had cooled, 9 wells 2 mm in diameter were made by suction using a needle connected to a vacuum tube. Nine 2 μL samples can thus be analyzed per 5 cm × 5 cm slab. After the electrophoresis (18 hours at room temperature, with a constant

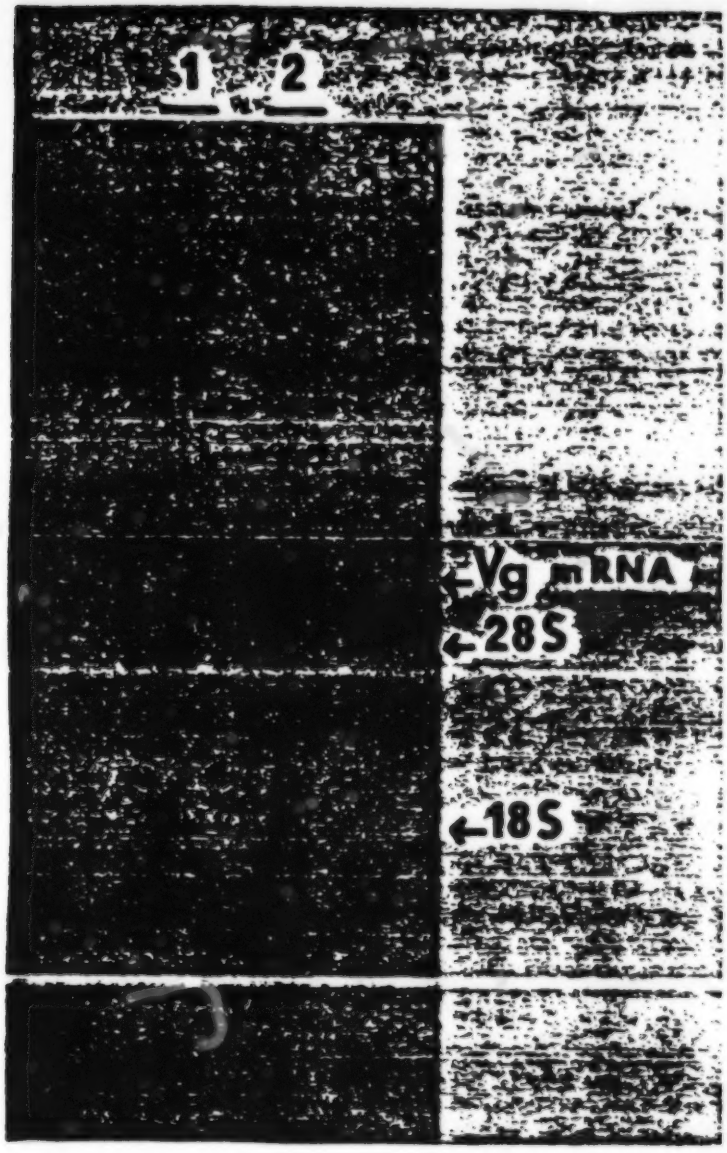
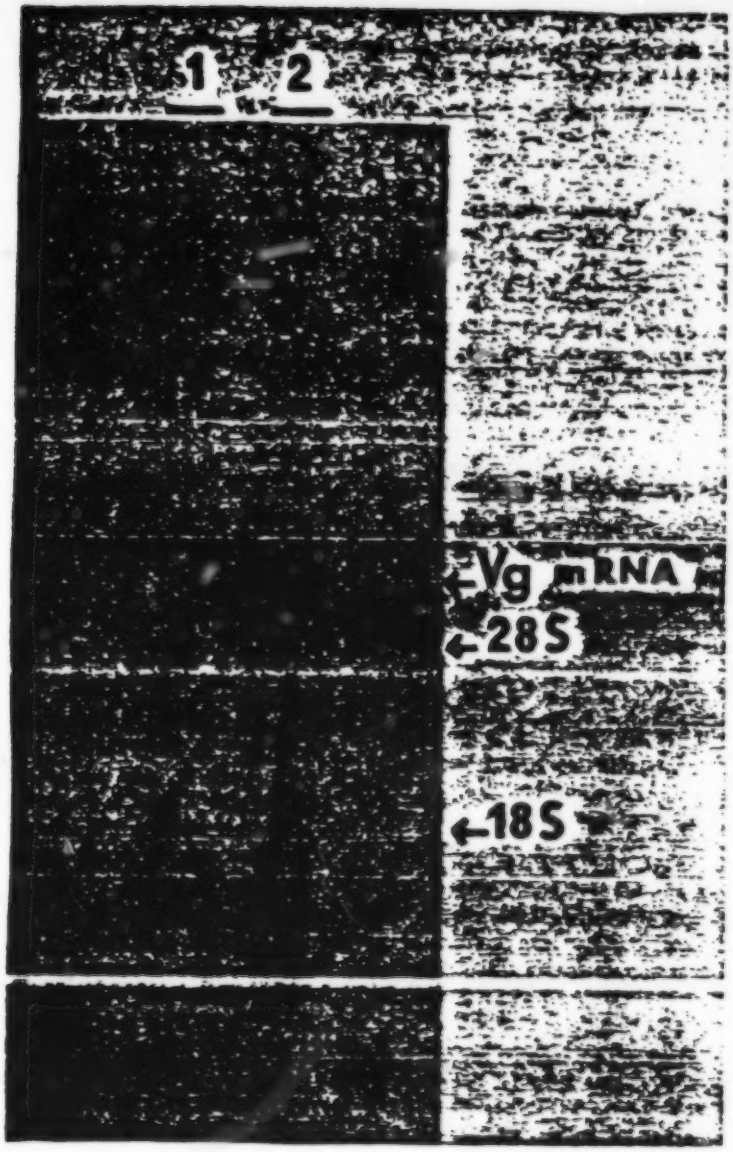


FIG. 1. Northern blot of total RNAs: 10 µg of total RNA from the livers of control (1) or estradiol-stimulated (2) male trout was transferred onto paper activated with aminothiophenol after this RNA was denatured and electrophoresed on 1.2% agarose gel.

Next, hybridization with the ^{32}P labelled vitellogenin probe was carried out on the paper under the described conditions; autoradiography was then conducted (24 hours of exposure at -80°C, Kodak X-Ray Film DEF 5). The positions of 28S and 18S RNAs visualized on the same gel by ethydium bromide are shown by the arrows.



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voltage of 2.3 volts/cm), the slabs were washed for 24 hours in 0.9% NaCl and for 1 hour in distilled water. They were then dried on Whatman 3MM paper in an oven at 40°C and stained with Coomassie blue. The amount of vitellogenin was determined by comparing the height of the immunoprecipitation peak for vitellogenin with the standard peaks obtained for increasing amounts of purified vitellogenin.

Dosage of the estradiol

This was carried out by radioimmunoassay (RIA) in the Laboratoire de Physiologie des Poissons [24].

RESULTS

Determination of the characteristics of the soluble 17β-estradiol receptor

Incubating the (6,7-^{3}H)-E_2 ± a 100-fold excess of (^{1}H)-E_2 with the cytosol at 0°C demonstrates specific binding of the 17β-estradiol in the trout liver cytosol. This binding is maximal after 30 minutes and is stable for at least 24 hours (L Dolo and L Mercier, unpublished results).

The parameters of 17β-estradiol binding in the cytosol were measured after incubation at 0°C for 3 hours. Under these conditions, no alterations of the (6,7-^{3}H)-E_2 were observed (Fig. 2).

The Scatchard plot for the binding of 17β-estradiol in the cytosol is not always linear, suggesting that there are at least 2 classes of binding site. Conversely, after $(NH_4)_2SO_4$ precipitation, a single high affinity ($K_D = 1.52 \times 10^{-9}$ M) component is found in the 30% AS fraction. The fractionated precipitation of the cytosol proteins by $(NH_4)_2SO_4$ allows the high affinity sites (30% AS fraction) to be separated from the low affinity sites (50% AS fraction), and the estradiol receptors to be partially purified. The number of binding sites in the 30% AS fraction ($n = 1.69 \times 10^{-13}$ moles/mg of protein) is indeed greater than that measured in the total cytosol ($n = 5.81 \times 10^{-14}$ moles/mg of protein) (Fig. 3).

Figure 4 shows the high specificity of the binding of 17β-estradiol. Of the steroids used, only the estrogens ((^1H)-E_2 and DES) are effective competitors of (^3H)-E_2. The relative affinity of DES, however, is lower (3-7 times) than that of E_2. 5α-DHT is a weak competitor, whereas 3β-diol, progesterone and dexamethasone are not competitors.

Induction of vitellogenin messenger RNA

Fifteen male trout, each weighing approximately 200 g, were divided into two groups: the first received an intraperitoneal injection of 17β-estradiol in saline solution (0.5 mg/kg body weight); the second received only solvent. This protocol was repeated every 2 days for 5 days, and the

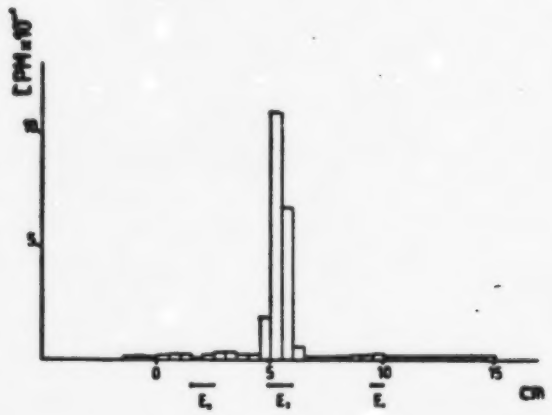
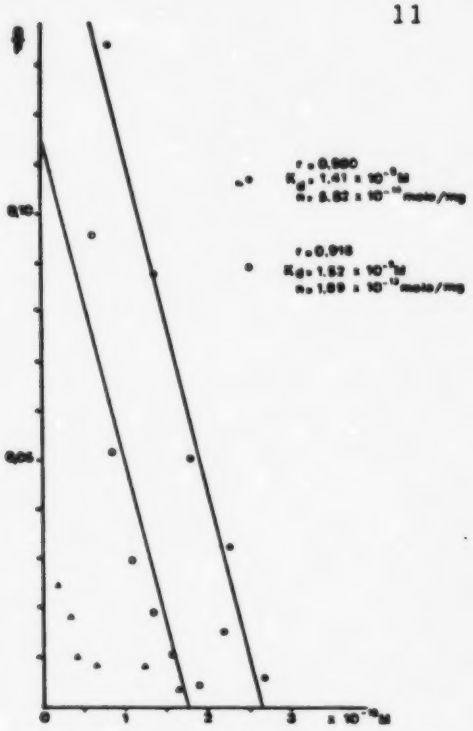


FIG. 2. Separation of steroids extracted after incubation of the cytosol at 0°C for 3 hours in the presence of ($6,7$-^{3}H)-E_2 (5×10^{-9} M). This separation involved silica gel chromatography carried out in a chloroform-ethyl acetate (80:20) system.

The positions of the control steroids are indicated by the horizontal lines under the X-axis (E_3=estriol, E_2=estradiol, E_1=estrone).



specific binding

FIG. 3

FIG. 3. Scatchard plot for the specific binding of (^{3}H)-E_2 in the cytosol.

Aliquot fractions of crude cytosol (-●-) (1242 µg protein/assay) or of preparations of partially purified cytosol receptors obtained after fractionated precipitation by $(NH_4)_2SO_4$, 30% AS fraction (-o-) (287 µg protein/assay) and 50% AS fraction (Δ) (525 µg protein/assay), were incubated at 0°C for 3 hours in the presence of increasing concentrations (7.5×10^{-10} M to 5×10^{-8} M) of (^{3}H)-E_2 ± a 100-fold excess of (^{1}H)-E_2. The amount of bound radioactive hormone was determined after removal of the free hormone with DCC. In all cases, the specifically bound hormone was determined by subtracting the non-specifically bound hormone from the total bound hormone (r represents the correlation coefficient evaluated for each of the strait lines; n represents the number of binding sites per mg of protein).

animals were sacrificed on the seventh day (protocol I). The repeated injections allow a considerable percentage of specific messenger RNA to be obtained.

Figure 5 shows the results of the hybridization of the poly $(A)^+$ RNA extracted from the livers of the 2 groups of fish and demonstrates that only the purified poly $(A)^+$ RNAs from the stimulated fish hybridize proportionately to the amount of RNA applied to the filters. Semi-quantitative estimates after 1.2% agarose electrophoresis or liquid phase hybridization had shown us that the poly $(A)^+$ RNA of stimulated animals contained approximately 10% vitellogenin mRNA [19]. The quantity of Vg mRNA that is detectable by the filter hybridization technique would therefore be of the order of 1 ng, which corresponds to the sensitivity previously determined by Searle et al. [25] for Xenopus vitellogenin mRNA.

Induction kinetics of vitellogenin messenger RNA

Fifteen male trout, each weighing approximately 200 g, received a single dose of 17β-estradiol (protocol II) under the same conditions as described previously. The fish were then sacrificed over time. Ten micrograms of total RNA from each sample were transferred onto the filters and hybridized to the specific probe.

Figure 6 shows that vitellogenin messenger RNA was induced after a 5-hour latent period and that the RNA level

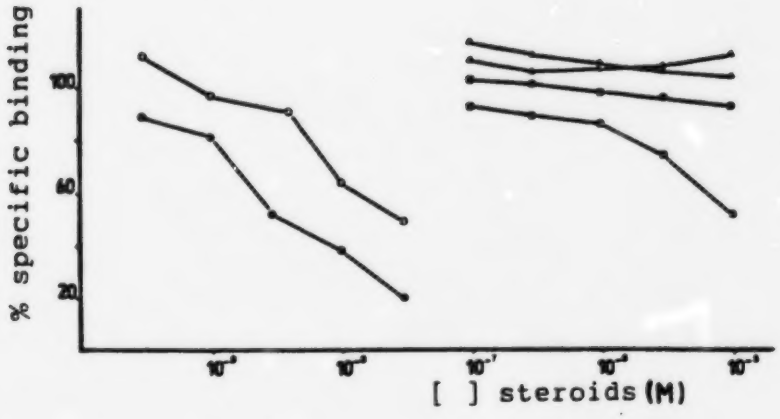


FIG. 4. Competition of (^{1}H)-E_2 (-●-), DES (-o-), 5α-DHT (-□-), 3β-diol (-■-), progesterone (-▲-), dexamethasone (-△-) with (^{3}H)-E_2 binding in the cytosol.

Fractions of cytosol were incubated in the presence of 1.60×10^{-9} M of (^{3}H)-E_2 and increasing concentrations of different steroid competitors, for 3 hours at 0°C. The bound radioactive hormone was determined after treating the fractions with DCC. Nonspecific binding was measured under the same conditions, in the presence of an 800-fold excess of (^{1}H)-E_2. The amount of specifically bound (^{3}H)-E_2 in the presence of different steroids is represented as a percentage of the binding of the control in the absence of competitor.

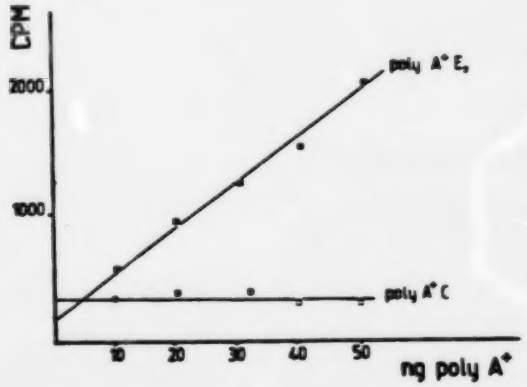


FIG. 5. Hybridization of poly $(A)^+$ RNAs from male trout stimulated with estradiol and from control trout (Protocol I).

Increasing amounts of poly $(A)^+$ RNA (10 to 50 ng) were applied to squares of Whatman 540 paper that were activated with aminothiophenol and diazotized. After hybridization with a tritium-labelled probe, the discs were burned in an "auto-oxidizer" (Packard Tricarb model 306) and the radioactivity was determined by liquid scintillation counting using an Isocap 300 counter (Nuclear Chicago).

(-■-) poly $(A)^+$ RNA of stimulated fish

(-□-) poly $(A)^+$ RNA of control fish

reached a maximum 48 hours after stimulation. This level then stayed constant for 5 to 6 days. Fifteen days after the injection, the mRNA level had already very clearly decreased: only 10% of the maximum induced quantity remained.

Quantitative determination of vitellogenin

Figure 7 represents a standard curve for quantifying vitellogenin by immunoelectrophoresis. Increasing amounts of purified vitellogenin (30 to 400 ng) were deposited in wells and electrophoresed in the presence of antibodies. The photograph, taken after the immunoprecipitation peaks were stained, shows that the height of the peaks (in mm) is directly proportional to the quantity of deposited vitellogenin. The lower dosage limit in trout serum is 10 µg/mL.

Induction kinetics of vitellogenin

The fish that were stimulated weighed roughly 1 kg so that blood could be sampled frequently enough (trout A: 1.2 kg, trout B: 0.8 kg). The 17β-estradiol was dissolved in cocoa-butter at 40°C and administered by intraperitoneal injection so that an implant would be produced when the cocoa-butter solidified (trout A: 3 mg estradiol per 1 mL of cocoa-butter, trout B: 0.05 mg per 1 mL of cocoa-butter). The estradiol concentration in the serum was measured by radioimmunoassay (RIA) [24]. Figure 8A shows that E_2 appears rapidly

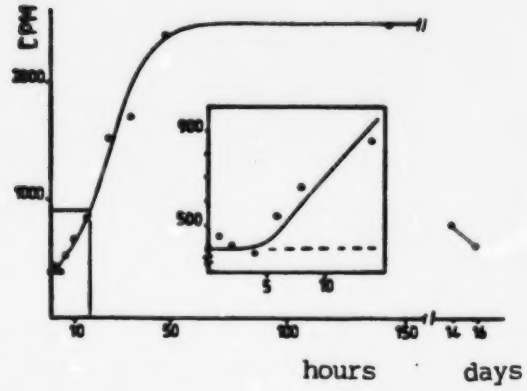


FIG. 6. Appearance kinetics of vitellogenin messenger RNA after stimulation with 17β-estradiol.

A group of 15 male trout, each weighing approximately 200 g, was stimulated with an intraperitoneal injection of 17β-estradiol solution (0.5 mg/kg body weight) (Protocol II). The animals were sacrificed at various times. Ten micrograms of total RNA were applied to the filters and the hybridization was determined by counting for radioactivity as described previously. The inset shows the kinetics soon after stimulation.

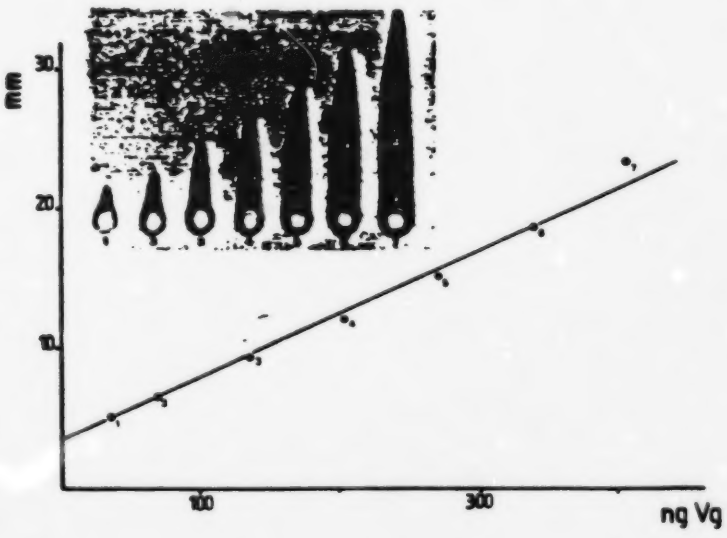


FIG. 7. Standard curve for vitellogenin.

Increasing quantities of vitellogenin, from 34 to 408 ng, were deposited into wells in 0.1% agarose gel containing antivitellogenin antibody at a dilution of 1/400. The migration was performed at 2.3 mvolts/cm for 18 hours. After the slabs were dried and stained, the immunoprecipitation peaks were measured and the height (mm) was expressed as a function of the quantity (ng) of vitellogenin.

in the serum of trout A and that its concentration is of the same order as that found in females during vitellogenesis (>10 ng/mL) [24]. A regular decrease in estradiol levels is observed over 25 days. In trout B (Fig. 8B), the estradiol concentration in the serum was very low. The only positive measurement was found the day after the injection. On this day the E_2 concentration was 1 ng/mL. This concentration is lower than that found in female trout during previtellogenesis [24]. The appearance kinetics of vitellogenin in the 2 fish were quantitatively very different. In trout A (Fig. 8A), vitellogenin could be detected in the serum after 24 hours of stimulation, and its concentration increased regularly, levelling off on the fifteenth day. At this time vitellogenin accounted for over half of the total proteins. In addition, it can be seen that, once reached, the maximum vitellogenin level persisted at least until the twenty-fifth day, although the estradiol concentration in the serum was considerably lower. In trout B (Fig. 8B), the vitellogenin concentration in the serum was very low, the maximum being approximately 500 times lower than in trout A. Nevertheless, the immunoelectrophoresis technique allowed us to follow the appearance kinetics of vitellogenin with precision, and it showed that concentrations of estradiol of the order of ng/mL of serum are sufficient to induce vitellogenin synthesis.

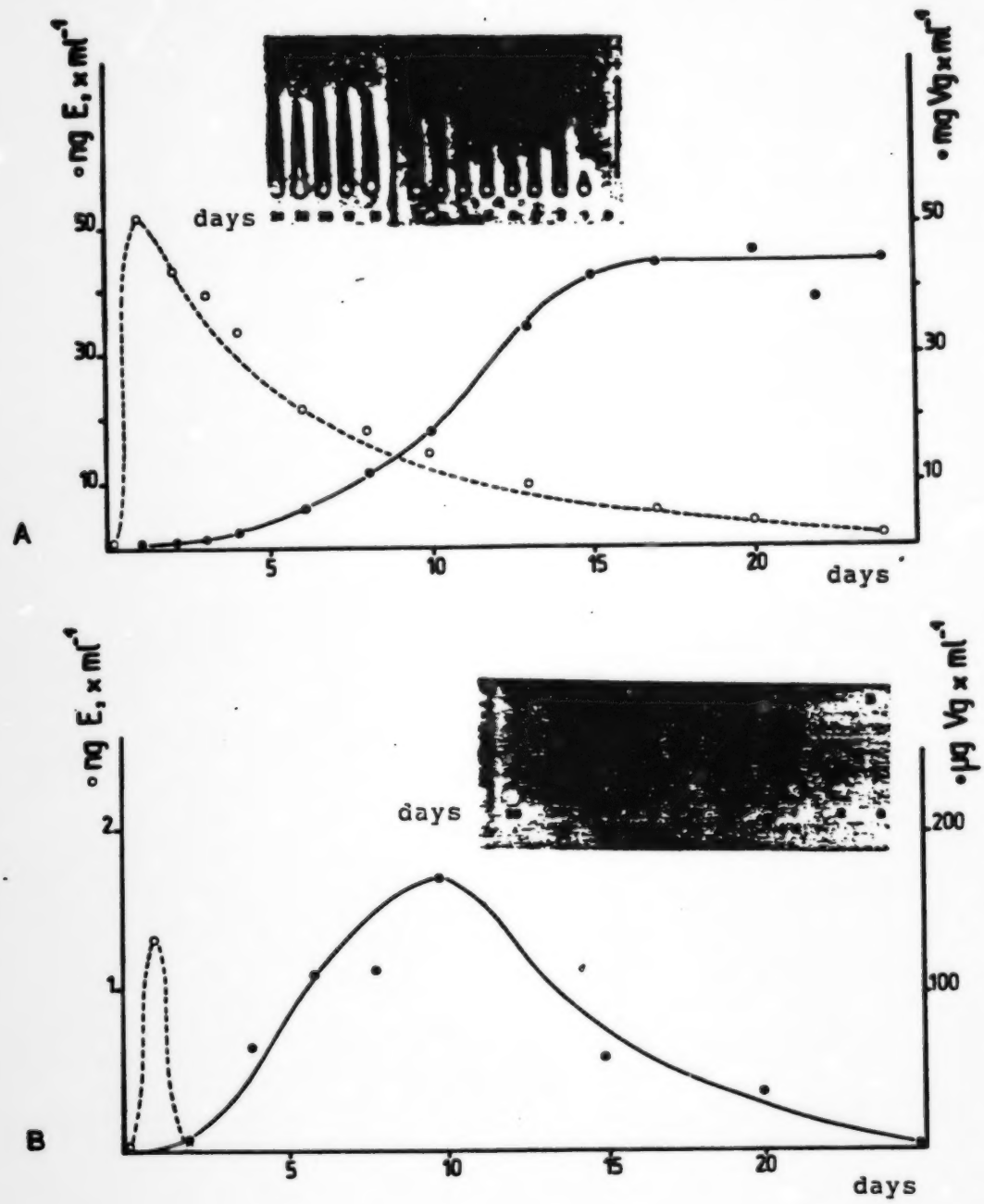


FIG. 8. Appearance kinetics of vitellogenin in the serum of male trout after the administration of 17β-estradiol.

The vitellogenin level in the serum was determined by immunoelectrophoresis using purified vitellogenin as a standard (see Fig. 6). Different dilutions of serum were utilized. --o-- estradiol, -●- vitellogenin.

7A: Stimulation with an estradiol implant (3 mg/mL). On the days indicated for stimulation, the following dilutions of serum were used in the dosage: 0, 1, 2 (1/4) - 3 (1/10) - 4 (1/20) - 6 (1/50) - 8, 10 (1/100) - 13 to 24 (1/200).

7B: Stimulation with an estradiol implant (0.05 mg/mL). All the sera were diluted by ½ before dosage.

DISCUSSION

Our results demonstrate the presence of estradiol receptors in the liver of male trout under conditions (incubation of the cytosol at 0°C) where this hormone is not metabolized.

High affinity binding sites ($K_D \sim 1.5 \times 10^{-9}$ M) were found in the crude cytosol and in preparations of receptors that were partially purified by precipitation in $(NH_4)_2SO_4$ at 30% saturation. Heterogeneity in the binding sites was sometimes observed in the crude cytosol. The Scatchard plots are resolved as two straight lines, indicating the presence of at least 2 classes of binding site. The significance of the second class of binding site is not easy to determine. The site may be an "unusual" protein binding the sex steroids, as described by Dickson et al. [26] in the livers of male rats, or even a plasma protein. It should be kept in mind that the liver is the site of plasma protein synthesis and that the rate of synthesis may vary according to the physiological state of the animal. Since the cytosol preparations do not come from animals in the same physiological state (different age, sperm producing or not sperm producing), this may explain the variation in the level of the second component. When the cytosol contains only one high affinity component, the dissociation constant is found to be identical to that measured in the 30% AS fraction ($K_D \sim 1.5 \times 10^{-9}$ M).

The binding of the hormone is very highly specific since only the estrogens E_2, DES, E_1 and E_3 (results not shown) are competitors. Unlike the other systems studied, the mammalian liver [27, 28], the mammalian anterior hypophysis [29, 30] and the fish liver [31,32], the relative affinity of DES in this case is 3 to 7 times weaker on average than that of E_2. DES is a synthetic compound that binds with low affinity or not at all to the sex hormone binding globulin (SHBG) [33]. Consequently, this lower binding affinity of DES for E_2 binding sites in trout liver cytosol raises some questions. Are the high affinity binding sites characteristic of specific receptors or are they the result of contamination by SHBG? The latter hypothesis, however, seems unlikely. Indeed, SHBG has also been found in the thorny skate, Raja radiata [34, 35], in the lesser spotted dogfish, Scyliorhinus canicula [36], and in the spiny dogfish, Squalus acanthias [37]. These authors describe SHBG as a protein that binds steroids at carbon atoms 18 (androgens), 19 (estrogens) and 21 (corticosteroids and progesterone) with high affinity. In the trout, Salmo gairdneri , Fostier and Breton [38] also described the presence of plasma proteins binding sex steroids and corticosteroids.

The high specificity of the binding sites for the estrogens only (very weak competition with 5α-DHT, no competition with

3β-diol, progesterone, and dexamethasone) seems to rule out the possibility that these sites are the result of contamination by plasma proteins, whose specificity is on the contrary very low.

By using hepatocyte preparations freshly isolated by collagenase perfusion of the liver, we were able to show that the characteristics of the E_2 receptors were identical to those of liver receptors ($K_D \sim 1.5 * 10^{-9}$ M) (unpublished results). We will now attempt to show that the characteristics of the receptors are also conserved in isolated hepatocytes maintained in primary culture, and that under conditions where the E_2 level is carefully controlled, there is a close correlation between the number of occupied binding sites, the level of vitellogenin messenger RNA, and the amount of synthesized vitellogenin.

The presence of specific vitellogenin messenger RNA could only be demonstrated in male trout after estradiol stimulation. In the control animals no molecular hybridization with the specific probe could be obtained, even after the quantity of poly $(A)^+$ RNA was increased from 5 to 50 ng. This result supports previous observations [19, 14]. The rainbow trout can therefore also be studied as an excellent model for the regulation of gene expression under hormonal control. Indeed, estradiol activates a gene that is normally

not expressed in the male, which is rather unusual in most of the models now being studied. We also have shown that the induction kinetics, as in other species, Xenopus, chicken [39, 40], start only after a latent period of 4 to 5 hours (inset Fig. 6). This latent period, which is more-or-less long according to the species, may result from the synthesis of specific receptors that persist at high levels even after the clearance of the injected estradiol [41, 42].

With respect to the appearance kinetics of vitellogenin, the estradiol implants used delivered hormone into the serum at levels comparable with those found in females during the normal cycle. It can thus be shown that serum estradiol concentrations as low as 1 ng/mL can induce vitellogenin synthesis in males; it may also be possible that vitellogenin synthesis in females starts earlier in the annual cycle than is currently believed.

These preliminary experiments were mainly intended to prepare the technological means needed to study the expression of the vitellogenin gene in the rainbow trout. We are now considering studying problems of a more physiological nature. A comparative *in vivo* study of the kinetics of vitellogenesis (receptors, messenger RNA, vitellogenin) in the 3 lines of *Salmo gairdneri* that lay their eggs at different times should allow us to determine whether vitellogenesis is induced synchronously, with subsequent modulation in the rate of

vitellogenin synthesis or if vitellogenesis is induced at different times, and the rates of synthesis are identical.

During the normal cycle, the induction of vitellogenesis is associated with an increase in the serum estradiol level [11, 12, 24]. At the same time, the level of estrone increases considerably. The role of this hormone in vitellogenesis has been demonstrated by Van Bohemen [11, 12]. We hope to obtain more accurate information on its mechanism of action by determining its effect on the different stages of regulation of vitellogenesis.

Finally, using a system involving isolated hepatocytes that we have developed (unpublished results), we hope to conduct in vitro testing of endogenous factors (hormones) and exogenous factors (antibiotics, herbicides, pesticides) that may influence vitellogenin production.

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